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Unravelling the removal mechanisms of bacterial and viral surrogates in aerobic granular sludge systems

Mary Luz Barrios-Hernández[a,b,c,*], Carolina Bettinelli[a], Karen Mora-Cabrera[d], Maria-Clara Vanegas-Camero[a], Hector Garcia[a], Jack van de Vossenberg[a], Daniel Prats[d], Damir Brdjanovic[a,b], Mark C M van Loosdrecht[b], Christine M Hooijmans[a]

[a] Department of Environmental Engineering and Water Technology, IHE-Delft Institute for Water Education, P.O. Box 3015, 2601 DA Delft, the Netherlands
[b] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, the Netherlands
[c] Escuela de Química, Instituto Tecnológico de Costa Rica, Cartago, 159-7050, Costa Rica
[d] Institute of the Water and the Environmental Sciences, University of Alicante, 03690, Alicante, Spain

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ABSTRACT

The aerobic granular sludge (AGS) process is an effective wastewater treatment technology for organic matter and nutrient removal that has been introduced in the market rapidly. Until now, limited information is available on AGS regarding the removal of bacterial and viral pathogenic organisms present in sewage. This study focussed on determining the relation between reactor operational conditions (plug flow feeding, turbulent aeration and settling) and physical and biological mechanisms on removing two faecal surrogates, *Escherichia coli* and MS2 bacteriophages. Two AGS laboratory-scale systems were separately fed with influent spiked with 1.0×10^6 CFU/100 mL of *E. coli* and 1.3×10^8 PFU/100 mL of MS2 bacteriophages and followed during the different operational phases. The reactors contained only granular sludge and no flocculent sludge. Both systems showed reductions in the liquid phase of 0.3 Log_{10} during anaerobic feeding caused by a dilution factor and attachment of the organisms on the granules. Higher removal efficiencies were achieved during aeration, approximately 1 Log_{10} for *E. coli* and 0.6 Log_{10} for the MS2 bacteriophages caused mainly by predation. The 18S sequencing analysis revealed high operational taxonomic units (OTUs) of free-living protozoa genera *Rhogostoma* and *Telotrochidium* concerning the whole eukaryotic community. Attached ciliates propagated after the addition of the *E. coli*, an active contribution of the genera *Epistylis, Vorticella,* and *Pseudovorticella* was found when the reactor reached stability. In contrast, no significant growth of predators occurred when spiking the system with MS2 bacteriophages, indicating a low contribution of protozoa on the phage removal. Settling did not contribute to the removal of the studied bacterial and viral surrogates.

1. Introduction

The aerobic granular sludge (AGS) process has been shown to be an effective technology for wastewater treatment (Bengtsson et al., 2018; Nancharaiah and Kiran Kumar Reddy, 2018). The current full-scale AGS technology (Nereda®) operates as an up-flow, anaerobically fed sequencing batch reactor (SBR) with simultaneous feeding and effluent withdrawal and in between an extended aeration period (Pronk et al., 2015b; de Sousa Rollemberg et al., 2018). The biomass in the AGS system consists of agglomerated bacteria that simultaneously remove organic matter and nutrients (de Kreuk et al., 2007). This agglomeration is possible due to microbial extracellular polymeric substances that bind individual cells into granules (Liu and Tay, 2002; Lin et al., 2010; Shi and Liu, 2021). The bacterial community in the granule consists of phosphate accumulating organisms (PAOs), ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) (Winkler et al., 2013; Szabó et al., 2017). These bacterial groups are responsible for organic matter and nutrient removal. Other organisms present, such as filamentous bacteria, protozoa and metazoans are related to the granule formation and system performance (Schwarzenbeck et al., 2004; Weber et al., 2007). Full-scale AGS systems contain a large granular sludge and smaller flocculent sludge fraction (Ali et al., 2019).

* Corresponding author. Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, the Netherlands
E-mail address: M.L.BarriosHernandez@tudelft.nl (M.L. Barrios-Hernández).

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In general, AGS wastewater treatment systems report high removal efficiencies of carbon, nitrogen and phosphorus (Pronk et al., 2015b; Pronk et al., 2017; Bengtsson et al., 2018). Besides a good treatment performance concerning the water quality parameters, two recent studies showed the capability of the AGS technology in removing bacterial and viral indicator organisms from sewage. Barrios-Hernández et al. (2020b) and Thwaites et al. (2018) compared removal efficiencies in AGS and conventional aerobic sludge (CAS) full- and pilot-scale wastewater treatment plants. The AGS full-scale systems can just as effectively remove indicator organisms as the CAS process. For example, the Log_{10} removal for both systems ranged between 1.7 and 2.6 for bacteria as *E. coli*, and between 1.4 and 2.4 for F-specific RNA bacteriophages. Both studies mentioned above emphasised that the presence of the variety of protozoa commonly present in wastewater treatments could be influencing the removal of the indicator organisms.

For a good understanding of pathogen removal by AGS systems, more mechanistic studies are needed. A large number of studies can be found for other wastewater treatment systems looking at biological (cell lysis and predation) and physical (adsorption and precipitation) removal mechanisms of pathogenic bacteria. A study by van der Drift et al. (1977) postulated that the faecal surrogate *E. coli* was either biologically predated by protozoa or ended up enmeshed into the sludge flocs. Hereafter, other researchers confirmed the importance of protozoa as grazers in CAS systems (Curds, 1982; Mallory et al., 1983; Madoni, 1994); and their role as primary predator during aeration (Curds, 1973). More recently, the removal of viruses in CAS systems has been studied, using bacteriophages as a surrogate for viruses, showing that their elimination from sewage can be challenging due to their persistence and abundance (Lucena et al., 2004; Amarasiri et al., 2017). Bacteriophages tend to either attach or detach from surfaces depending on the surrounding water conditions (Bales et al., 1993). They can also be predated by heterotrophic flagellates (González and Suttle, 1993; Deng et al., 2014). According to Stevik et al. (2004) and Dias et al. (2017), their retention and depletion in wastewater may be affected by system configuration, hydraulic retention time, water quality (temperature, pH and organic matter), and water flow velocity, among other factors.

It is still unclear which removal mechanism plays a major role in AGS systems, especially in the granular fraction, and how far these mechanisms are linked to the different operational phases. The main goal of this study was to relate the operational conditions of an AGS laboratory-scale reactor with the removal of a faecal bacterial surrogate *E. coli* and a viral surrogate MS2 bacteriophage. Moreover, the attachment of the faecal organisms onto the granules, protozoa predation and the contribution of the settling in the bacterial and viral surrogate removal process was studied.

2. Materials and methods

2.1. Research design

Two laboratory-scale reactors were operated long-term as sequencing batch reactors (SBR). Both systems developed a steady-state situation with mature granules when fed with only synthetic wastewater. When the steady-state was reached, the influent was spiked with known concentrations of two typical surrogates for bacterial and viral water quality, *E. coli* bacteria and MS2 bacteriophages. Reactors were monitored weekly for physicochemical and microbiological water quality parameters before and after each cycle operational phase (anaerobic plug feeding, aeration phase and settling). Changes in the protozoa community were observed using microscopy observation, and changes in the eukaryotic community were studied using 18S rRNA sequence analysis. Next to the long-term investigation, additional batch experiments were executed to better understand predation (using a fluorescent staining technique) and attachment of the surrogates on the granular surface. The contribution of the settling phase to the removal of the studied surrogates was also evaluated.

2.2. Laboratory-scale SBR

Two laboratory-scale SBRs (hereafter called AGS_*E. coli* and AGS_MS2) were operated for 154 and 125 days, respectively. The operational cycles follow the sequence of an anaerobic phase, aeration (reaction) phase, and settling and effluent withdrawal (Figure 1). During the anaerobic phase, the systems were fed in a plug-flow mode for 60 min. Hereafter, an air recirculation pump operated at 6 L/min kept the system completely mixed and aerated (1.8 mg/L) for 110 min. A 5 min settling time was followed by effluent discharge, creating a sludge selection mechanism resulting in dense granules in the reactors.

The reactors consisted of a double-wall glass bubble column of 2.9 L (CBN, the Netherlands). They were operated and controlled with a Braun DCU4 controller, coupled with both mass-flow and a multi-fermenter control system (MFCS), using acquisition software (Santorious Stedim Biotech S.S., Germany). The system was operated and controlled with an Applikon ADI controller model 1030, connected to a computer with the software BioXpert 2 (Applikon, the Netherlands). Both systems were operated at 20 ± 1 °C. The pH of 7.0 ± 0.1 was automatically controlled by adding either 1M HCl or 1M NaOH. Both systems were inoculated with crushed sludge from an AGS full-scale WWTP (Garmerwolde, the Netherlands) with an initial total suspended solids (TSS) concentration of 8.3 ± 2.5 g/L.

2.2.1. Synthetic wastewater

The systems were fed with the synthetic wastewater composed of acetate (2.9 kg/m^3/day), ammonium-nitrogen (0.48 kg NH_4-N/m^3/day), phosphorus source (0.08 kg PO_3-P/m3/day) and trace metals prepared according to the Vishniac and Santer (1957)' solution. The bacterial and viral surrogates were added once the granular stability in the reactors was established, i.e., after day 47 in the AGS_*E. coli* reactor and after day 69 of operation in the AGS_MS2 reactor. The concentration in the influent for the AGS_*E. coli* reactor was between 1×10^4 and 1×10^7 CFU/100 mL. For the AGS_MS2 reactor, concentrations were between 1×10^5 and 1×10^8 PFU/100 mL.

2.2.2. Bacterial and viral surrogates

Due to their importance for water quality regulations, two faecal surrogates were selected and enumerated, as explained in Scoullos et al. (2019).

2.2.2.1. E. coli *strain, culture and enumeration.* The *E. coli* ATCC reference strain 25922 was taken as bacteria surrogate for faecal contamination. *E. coli* was initially inoculated in a sterilised Nutrient Broth medium (Merck KGaA, Germany) and incubated on a shaking platform (150 rpm) at 37 ± 1 °C for 24 hours. A stock concentration of about 1×10^9 CFU/100 mL was obtained and enumerated by spreading the medium on *Chromocult* (Sigma-Aldrich, Germany) coliform agar plates. Later, the suspension was spiked to the influent of the AGS_*E. coli* reactor by diluting the stock culture into a vessel of 10 L to an end concentration in the reactor of 1×10^7 CFU/100 mL. For enumeration, viable counts were conducted in triplicate, according to ISO 9308-1 (Anon 2000a). Aliquots of 0.1 mL of either pure or diluted sample were spread on the coliform agar plates and inoculated overnight at 37 ± 1 °C. Undiluted samples with expected concentrations lower than 30 CFU/100 mL were analysed in duplicate using membrane filtration. That is, 100 mL of

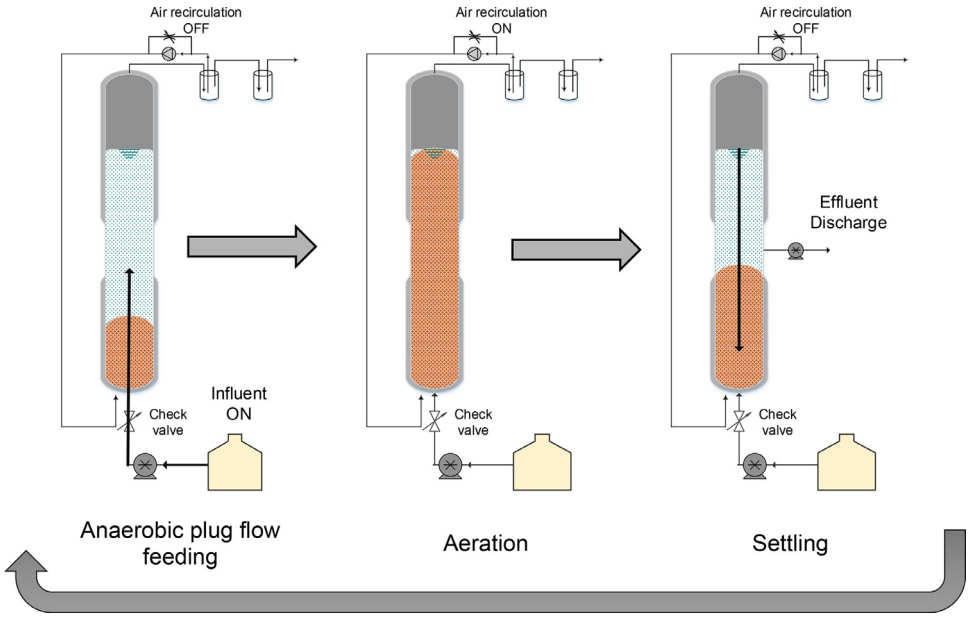

Figure 1. Cycle operational conditions of the AGS reactors.

the undiluted sample was passed through a cellulose nitrate membrane filters (0.45 μm). The filter was placed on *Chromocult* coliform agar plates and incubated at 37 ± 1 °C for 24 hours.

2.2.2.2. Bacteriophage strain, culture and enumeration. The *E. coli* bacteriophage MS2 reference strain ATCC 15597-B1 was used as the viral surrogate. The phage was propagated in Tryptone Yeast Glucose Broth (TYGB) using another *E. coli* strain ATCC 15597 as host bacteria while shaking at 150 rpm. The incubation temperature was 37 ± 1 °C for 24 hours to reach a stock concentration of 1×10^{12} PFU/100 mL. Working solutions were prepared in saline water buffer before being applied to the AGS_MS2 reactor by diluting the stock culture into a vessel of 10 L to a concentration 1×10^{8} PFU/100 mL. The MS2 bacteriophage enumeration was determined based on ISO 10705-1 (Anon 2000b) as plaque-forming units (PFU). The host bacteria (1 mL) was cultured in 50 mL of TYGB to a concentration of approximately 10^{8} PFU/100 mL. Samples diluted 10-fold, 100-fold and 1000-fold were mixed in semisolid Tryptone Yeast Glucose Agar (TYGA) and poured in solid TYGA before being incubated at 37 ± 1 °C for 18 hours.

2.3. Sample collection and processing

2.3.1. Physicochemical water quality parameters

For the performance of the reactor, 10 mL samples were taken from the liquid bulk before the aeration phase (62 min) and from the effluent. The samples were filtered through a 0.45 µm filter (Millex-HV, Germany) and subjected to the following analysis: chemical oxygen demand (COD), orthophosphate (PO_4-P) and nitrogen-related parameters such as NH_4-N, nitrite (NO_2-N), and nitrate (NO_3-N). For the COD measurement, the Closed Reflux-Colorimetric Standard Method (APHA, 2012) was used. The rest of the measurements were performed using LCK (Hach, Germany) cuvette tests. To control the optimal biomass growth, the TSS and volatile suspended solids (VSS) were determined according to the Standard Methods (APHA, 2012) for sludge samples and treated effluent samples.

2.3.2. Microbiological sampling process

For microbiological enumeration, samples were taken weekly from both reactors (AGS_*E. coli* and AGS_MS2) at the following sampling points: influent (10 mL), mixed liquor at the end of the anaerobic phase (25 mL), mixed liquor at the end of the aerobic phase (25 mL) and effluent (10 mL). From the mixed liquor samples, the sludge was separated from the liquid (hereafter referred to supernatant) by letting the sludge settle for 5 min. The supernatant (10 mL) was extracted with a syringe and placed in a separated vessel. Approximately 1 mL of the settled sludge fraction was crushed and homogenised using a glass/Teflon potter Elvehjem tube. All samples were enumerated in duplicate, as explained in Section 2.2.2. Results from the supernatant and sludge fractions after anaerobic and aerobic phases were subjected to a statistical analysis (Wilcoxon signed-rank test) after normalization to determine whether paired mean concentrations were significantly ($p < 0.05$) different from each other or not. The number of samples (n) for the analysis was between 8 and 13.

2.4. Optical microscope observation of protozoa

Additional samples of granules (5 mL) were taken during aeration to be inspected for protozoa presence. Samples of 25 µL were observed under optical microscopes Olympus CH30 (10x, 20x, and 40x) and Olympus BX51 (10x, 20x, and 40x). The stalked ciliated protozoa activity (occurrence and mobility) was studied based on a qualitative and quantitative scale observation of the individuals, as described in Amaral et al. (2018). For the 40x magnification, an area of approximately 37 mm^2 was measured, the highest value of 100% was assigned to the ones that showed high activity and more than six individuals/mm^2 in all the observations. A value of 5% was assigned to samples that at least showed one individual/mm^2 in any of the measured samples. Samples were checked in triplicates.

2.4.1. DNA extraction and 18S rRNA gene sequencing

Genomic DNA was extracted from approximately 0.25 g of crushed sludge collected on days 104 and 160 from the AGS_*E. coli* reactor, day 90 from the AGS_MS2 reactor, and the seed sludge using QIAamp PowerFecal PRO DNA kit (QIAGEN). The DNA concentration was determined using an Invitrogen Qubit Fluorometer (Thermo Fisher Scientific, USA). The V4 region of 18S rRNA genes was amplified using the following eukaryote-specific primers pair 528F 5′-GCGGTAATTCCAGCTCCAA-3' and 706R 5′-AATCCRAGAATTTCACCTCT-3′. PCR reactions were carried out with

Phusion High-Fidelity PCR Master Mix (New England Biolabs). 1x loading buffer (contained SYBR green) was mixed with the PCR products and run on 2% agarose gel electrophoresis. Products between 400bp-450bp were purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries were generated with NEBNext UltraTM DNA Library Prep Kit for Illumina (Illumina NovaSeq 2500, USA) and quantified via Qubit and Q-PCR. Paired-end reads were merged using FLASH (V1.2.7). Chimeras were removed using Qiime (Version 1.7.0), and sequences analysis were performed by Uparse software (Uparse v7.0.1001). Operational Taxonomic Units (OTUs) were obtained by clustering with $\geq$ 97% similarity. The analysis was performed using Silva database for species annotation. The raw sequence data were uploaded to the National Center for Biotechnology (NCBI) under accession numbers: SAMN16526359, SAMN16526360, SAMN16526361, and SAMN16526362.

2.5. E. coli *fluorescence microscopy observations*

To identify and record protozoa predation, a fluorescence staining detection method for *E. coli* was used. Granules from an additional AGS laboratory-scale reactor as well as granules from a full-scale WWTP were checked on the abundance of ciliates attached to the granular surface. The *E. coli* ATCC 25922 was labelled using a dsGreen gel staining solution 10,000x Lumiprobe (Hannover, Germany). It was analysed with the fluorescein isothiocyanate (FITC) filter set in the microscope. *E. coli* was 10-fold diluted as follows, 2 µL of the 10,000x dilution of dsGreen was added to a 1.998 µL of *E. coli* ATCC 25922 to obtain the final working solution of 1×10^4 CFU/ µL. Mini batch reactors were prepared in 2 mL Eppendorf tubes in which 1 mL of granules were spiked with 1 mL of the solution with the previously labelled *E. coli*. The solution was quickly mixed three times in a pulsing vortex mixer (VWR, Germany), then incubated in the dark for 15 minutes. The treated granules were washed three times in 400 µL of 1x phosphate-buffered saline (PBS) and centrifuged at 4,000 rpm for 5 min (Eppendorf MiniSpin, Germany); then resuspended in 1x PBS to get a final volume of 2 mL. Aliquots of 3 µL were placed on glass slides and analysed under an Olympus BX51 fluorescent microscope coupled with an XM10 camera, an X-cite fluorescence lamp (Lumen Dynamics, Series 120Q) and a FITC filter. Approximately between 8 and 12 sets of pictures of different visual parts of the granules were taken. For each picture set, both phase contrast and fluorescence images were taken at magnifications from 10 to 100x. Overlay pictures were analysed using Fiji image analysis software (https://fiji.sc/).

2.6. Attachment of E. coli *and MS2 bacteriophages*

To determine whether *E. coli* and MS2 bacteriophages attached to the granules, batch tests were performed at the same temperature as the long-term study (20 °C). Round-shaped granules (from 0.2 to 3.8 mm) from an additional control AGS laboratory-scale reactor were tested. The AGS reactor was fed only with synthetic wastewater. Therefore, there were no *E. coli* bacteria, MS2 bacteriophages, nor potential predators microscopically detectable, such as free-swimming and attached ciliated protozoa. The experimental tests were carried out based on Hendricks et al. (1979) with the following modifications. Three beakers were prepared with 50 g of the fresh granules and filled up to 200 mL with a synthetic wastewater solution. The beakers were mixed continuously with a magnet stirrer (250 rpm); then spiked with a known concentration of the target microorganisms (10^5 and 10^7 CFU/100 mL of *E. coli* bacteria, and 10^6 and 10^9 PFU/100 mL of MS2 bacteriophage). The initial concentration in the attachment test (C_0) was measured by taking 1 mL from the suspension (liquid bulk). The experiments aimed to analyse the behaviour of the surrogates during the 60 minutes of anaerobic feeding, therefore, 1 mL was consecutively taken from the suspension after letting the sludge settle at times (C_t) 5, 15, 30, 45 and 60 min. Since target organisms keep suspended in the liquid phase, the difference between C_0 and C_t was assumed to be caused by attachment to the granular media. Experiments were performed in duplicates, as well as the spreading and enumeration of the microorganism.

Kinetics were calculated using the pseudo-second order equation (Eq. 1) explained by Simonin (2016), in which t corresponds to the exposure time in minutes between the target organism and the granules, q is the *E. coli* bacteria (CFU/g) or the MS2 bacteriophages (PFU/g) concentration attached per gram of granule, q_e is the maximum attachment capacity of the organisms (CFU/g or PFU/g), and k is the fitted constant.

$$\frac{t}{q} = \left(\frac{1}{q_e}\right)t + \left(\frac{1}{kq_e^2}\right) \quad (1)$$

2.7. Contribution of the settling in the AGS reactor

The effects of the settling on the removal of the target microorganisms were independently evaluated in an additional AGS reactor, operated like the long term studied reactors but without being fed with any surrogate. The granules, cultivated only with synthetic wastewater, were spiked with either *E. coli* bacteria or MS2 bacteriophages and thoroughly mixed by aeration for 5 min to reach an equilibrium between supernatant and granules. The test was performed twice with different concentrations per indicator (around 10^5 and 10^7 CFU/100 mL for *E. coli* and around 10^3 and 10^9 PFU/100 mL for MS2) to determine how far the outcome of the test was affected by the concentration. At the end of the 5 min mixing, a sample was taken to determine the initial indicator concentration in the reactor. After turning off the aeration, a settling time of 5 min was allowed. After settling, the following samples of 5 mL were taken: treated supernatant at three different heights of the reactor column from the effluent discharge point (20, 40 and 60 cm), and a final mixed effluent sample. To separate the liquid fraction from the solids, the mixed sample before settling and the effluent sample were treated by let them settle for 5 min in a measuring cylinder. After that, microbial spreading and enumeration were carried out as described in Section 2.2.2.

3. Results

3.1. AGS reactors performance

The performance of two granular sludge reactors (AGS_*E. coli* and AGS_MS2) for the concentration of COD, PO_4-P, NH_4-N, NO_2-N, and NO_3-N are given in Figure 2. The reactors contained only granular sludge, while flocculent sludge was absent due to feeding with soluble substrate only.

The measurements showed good performance in terms of COD removal, see Figure 2a and Figure 2b. Concentrations were reduced at the end of the anaerobic phase from 402 ± 50 mg COD/L to averages of 40 ± 22 and 65 ± 17 mg COD/L after 47 and 57 days of operation in the AGS_*E. coli* and AGS_MS2 reactor, respectively. The effluent showed final average concentrations of 36 ± 21 and 34 ± 14 mg COD/ L, correspondingly. This effluent COD was mainly related to the non-biodegradable EDTA present in the influent. The systems also showed P-release with average values of 59 ± 16 mg PO_4-P/L for AGS_*E. coli* and 61 ± 22 mg PO_4-P/L for AGS_MS2. The phosphate removal was always good, with concentrations lower than 1 mg PO_4-P/L in the treated effluent for both reactors. Regarding nitrogen, the average ammonia-nitrogen concentration in

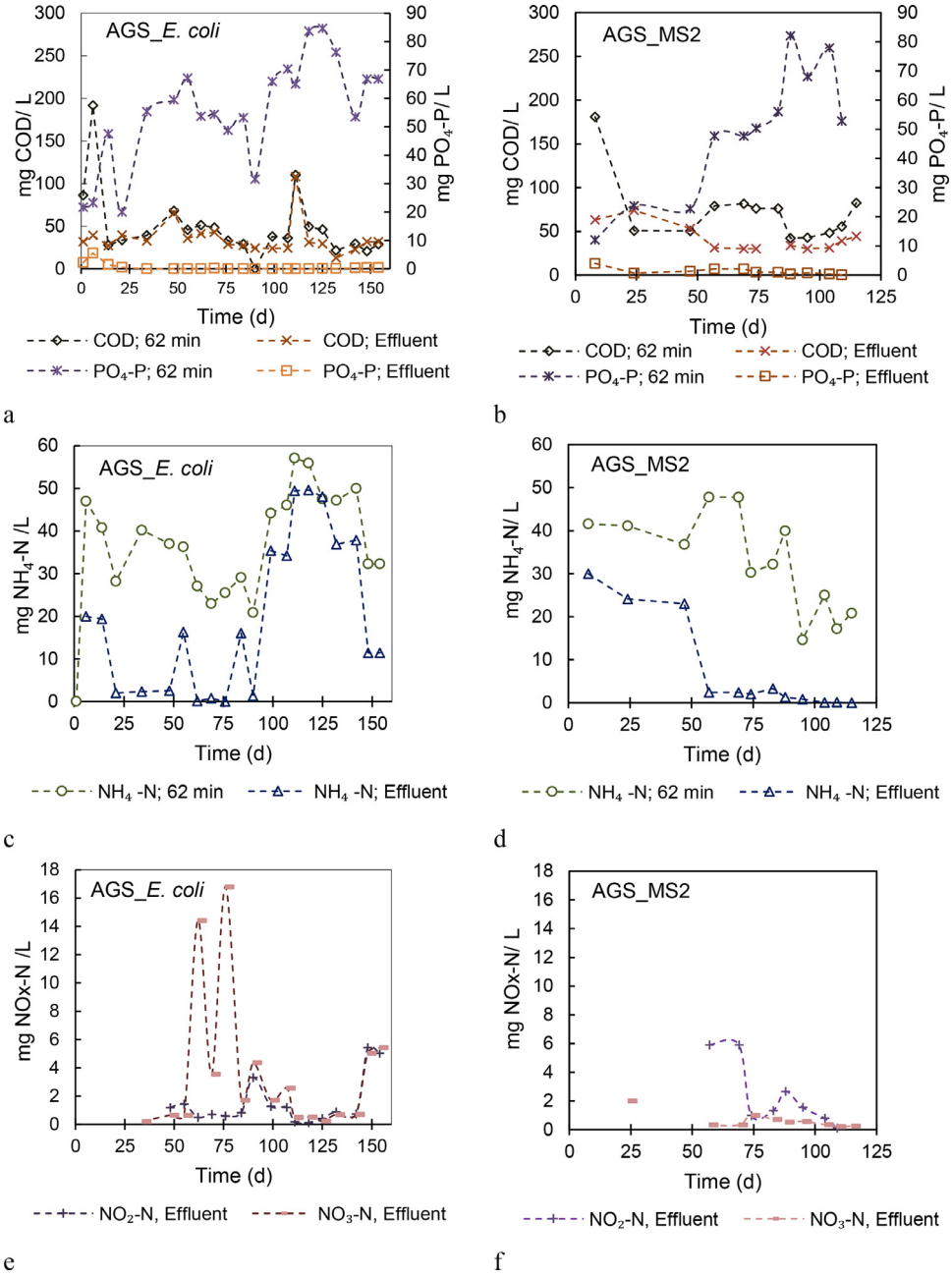

Figure 2. Performance of the laboratory-scale AGS reactors fed with *E. coli* (AGS_*E. coli*) or MS2 bacteriophages (AGS_MS2) for COD, phosphate and nitrogen removal.

the influent was 58 ± 13 mg NH_4-N/L, which was partially converted to NO_2-N and NO_3-N during the aeration phase. Effluent values were on average 20 ± 19 mg NH_4-N /L, 2 ± 2 mg NO_2-N /L, and 4 ± 5 mg NO_3-N /L for the AGS_*E. coli* reactor (Figure 2c and Figure 2e). For the AGS_MS2 reactor average concentrations of 8 ± 9 mg NH_4-N /L, 2 ± 2 mg NO_2-N /L, and 0.6 ± 0.5 mg NO_3-N /L were measured (Figure 2d and Figure 2f). It seems that copper from the feeding valve negatively affected the ammonia-oxidising bacteria community in the AGS_*E. coli* reactor. The valve was in use from day 90 to day 148. The dissolved oxygen was increased from 1.8 to 3.8 mg/L to stimulate the nitrification process. However, after replacing the valve, this was not necessary anymore. Oxygen was set at 1.8 mg/L; after which the system stabilised again. Since the behaviour of the N-conversion process was assumed not be influencing the removal of *E. coli* and MS2 bacteriophage the nitrification was not optimised.

3.2. Fate of the faecal surrogates in the long-term AGS laboratory-scale SBRs

3.2.1. Faecal surrogates removal

The measured median E. *coli* concentration in the influent was 1.0×10^6 CFU/100mL, ranging from 4.5×10^4 to 2.0×10^7 CFU/100 mL in the AGS_*E. coli* reactor. The MS2 bacteriophage influent concentrations for the AGS_MS2 reactor ranged between 4.0×10^5 and 7.5×10^8 PFU/100 mL, with a median of 1.3×10^8 PFU/ 100 mL. The effluent concentrations were between 1.0×10^2 and 7.1×10^5 CFU/100 mL for *E. coli* (median of 9.0×10^4 CFU/100 mL); and the MS2 bacteriophage concentrations were between 3.0×10^5 and 1.9×10^8 PFU/100 mL (median of 5.5×10^7 PFU/100 mL). The overall median of the *E. coli* and MS2 bacteriophage removal in the systems, comparing influent and effluent, was 2.2 and 0.3 Log_{10} (Figure 3a), respectively.

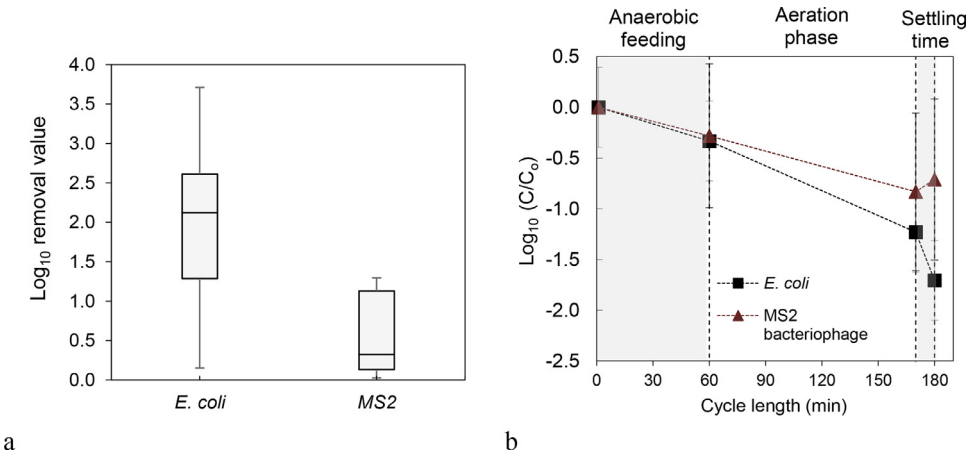

Figure 3. Overall average removal of the target surrogates *E. coli* bacteria (n =17) and MS2 bacteriophages (n = 9) in laboratory AGS reactors (a) and average depletion curves per operational cycle (b).

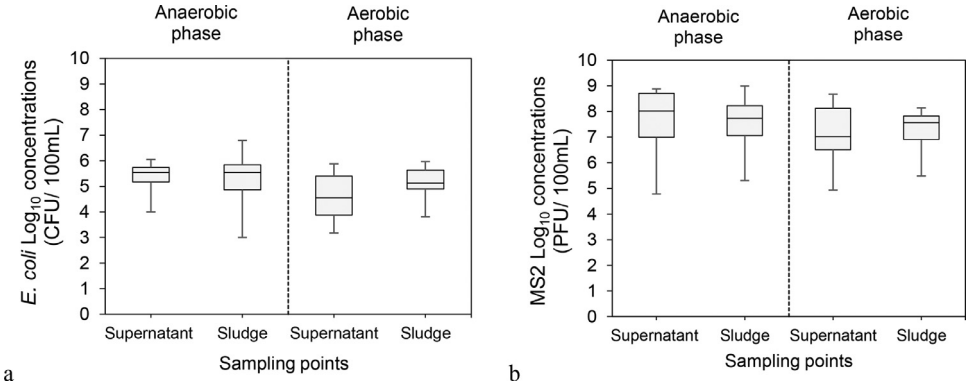

Figure 4. Log_{10} concentrations of *E. coli* bacteria (a) and MS2 bacteriophages (b) in the supernatant and granular fractions at the end of the anaerobic and aerobic operational phases.

Table 1
p-values obtained from the Wilcox test comparing concentrations observed in the supernatant and the sludge fraction after each anaerobic and aerobic operational phase.

Organism	Sampling point	n	W	*p*-value
E. coli	Supernatant Anaerobic-Aerobic phase	13	90	*0.0005*
	Granules Anaerobic-Aerobic phase	13	83	*0.0061*
MS2 bacteriophage	Supernatant Anaerobic-Aerobic phase	9	42	*0.0195*
	Granules Anaerobic-Aerobic phase	8	29	0.4961

E. coli and the MS2 bacteriophages removal profiles were fitted to a Chick–Watson model (Figure 3b) using the average concentrations measured in the influent (C_0) and the liquid bulk fractions (C); at the end of both the anaerobic phase and the aerobic phase and effluent. After the anaerobic phase (at time 62 min), a reduction of 0.3 Log_{10} was measured for AGS_*E. coli* and AGS_MS2 reactors. During aeration, the *E. coli* concentration decreased by 0.9 Log_{10}, whereas the MS2 bacteriophages decreased by 0.6 Log_{10}. After settling time, a further reduction of 0.5 Log_{10} was measured for *E. coli*. MS2 bacteriophage remained constant.

3.2.2. Surrogates concentrations in the sludge and liquid fractions per operational phase

Figure 4 shows the Log_{10} concentrations of the target microorganisms (*E. coli* bacteria and MS2 bacteriophage) measured after the fractionation of the supernatant and the granular sludge portion after the anaerobic/aerobic phases. Table 1 shows the *p*-values obtained from the comparison of the phases. At the end of the anaerobic plug flow feeding, the median of the *E. coli* concentration for both supernatant and the sludge fraction was 3.8×10^5 CFU/100 mL (or 5.5 Log_{10}) (Figure 4a). At the end of the aerobic phase (110 minutes of aeration), the *E. coli* median in the supernatant was reduced to 3.6×10^4 CFU/ 100 mL, or 4.6 Log_{10}, while the sludge fraction kept a more or less similar median concentration of 1.4×10^5 CFU/ 100 mL, or 5.1 Log_{10}). Both reductions were significantly different ($p << 0.05$) than in the previous anaerobic phase.

For the MS2 bacteriophage (Figure 4b), a median concentration of 1.0×10^8 PFU/100 mL (or 8.0 Log_{10}) was measured at the end of the anaerobic plug flow feeding in the supernatant. The median of the counts in the sludge fraction was 5.4×10^7 PFU/ 100 mL (or 7.7 Log_{10}). After the aeration phase, a significant difference ($p = 0.02$, Table 1) was observed in the supernatant portion with 1 Log_{10} unit reduction in the median counts (1.1×107 PFU/ 100 mL or 7.0 Log_{10}). In the sludge fraction, no significant differences ($p > 0.05$, Table 1) were observed when comparing the median of the aerobic phase (3.7×10^7 PFU/100 mL, or 7.6 Log_{10}) with the median of the previous anaerobic phase.

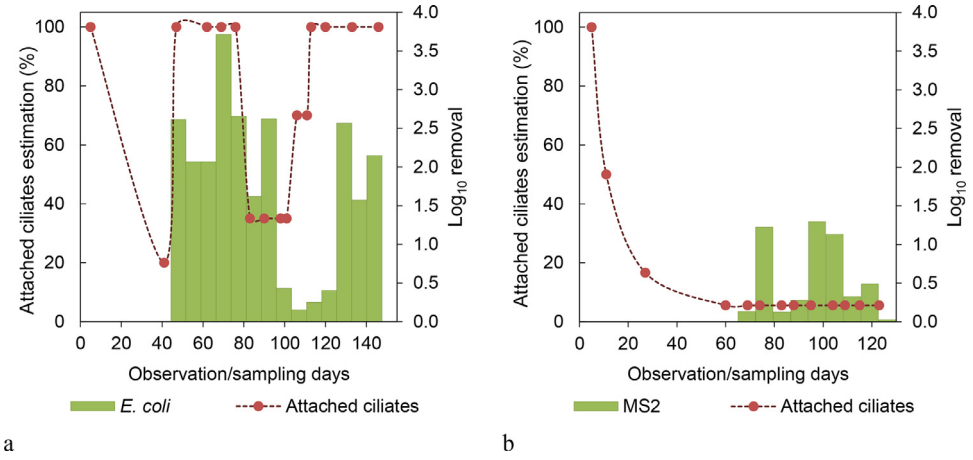

Figure 5. Attached ciliates abundance estimation (%) calculated based on the microscopic observations compared with the removals of *E. coli* bacteria (a) and MS2 bacteriophage (b).

3.3. Relationship of the protozoa relative abundance and the pathogen removal

Higher organisms typically present in the crushed granular sludge inoculum of the reactor were not microscopically observed when both reactors (AGS_*E. coli* and AGS_MS2) achieved their stable operation before adding *E. coli* and MS2. The stability was indicated by well-shaped granules that were formed and the accomplished stable COD and PO_4-P removal - after approximately two months. As soon as the reactors were inoculated with *E. coli* or MS2 bacteriophages, a sudden bloom of stalked ciliated protozoa attached to the granular surface was observed in the AGS_*E. coli* reactor, but not in the AGS_MS2 system. The attached ciliates occurrence in the granular samples was determined and compared with the removal of *E. coli* and MS2 (Figure 5). In the AGS_*E. coli* reactor, high activity (abundance and mobility) of attached ciliates was observed on days 48, 76, 113, and after 120. However, a decrease in the stalked ciliated activity occurred between days 86 and 105, which also coincided with both the reduction of the NH_4-N concentration shown in Figure 1c and a reduction of the *E. coli* removal in the system (Figure 5a). In contrast, in the AGS_MS2 reactor, no massive changes were observed for the stalked ciliated community when the system was spiked with the MS2 bacteriophage, coinciding with lower removals during the studied period.

3.3.1. Microbial community analysis

The eukaryotic microbial community was characterised by 18S rRNA gene analysis (Figure 6 and Figure 7). The analysis covered more than 99% sequencing depths (see Supplementary Materials, Table S1), which is sufficient to cover the whole community. The index used to estimate the number of the species (abundance) in a community belonging to individual classes, Chao1(Chao, 1984), showed that the number of species (richness) decreased compared to the seed sludge. That is from 818.3 in the seed sludge to 589.5 and 493.3 in the AGS_*E. coli* day 104 and day 160, respectively. At the same time, it remained almost the same (818.4) for the AGS_MS2 reactor (day 90). Instead, the Shannon diversity index used to determine the variation of living organisms (Kim et al., 2017) showed a reduction in the diversity in all the samples with values of 5.39, 2.03, 3.01, and 2.96, respectively. It confirms an expected reduction of the richness and evenness of the species from the seed sludge. The most abundant (top 10) species in all the studied samples are described at the phylum levels (Figure 6) of the eukaryotic phylogenetic classifications. Mostly free living organisms such as nematodes, tardigrades, and rotifers were likely removed via the effluent along with other particulate and suspended solids. Ascomycota and unidentified eukaryote were the most abundant groups found in the laboratory-scale systems.

Figure 7 summarises the most abundant protozoa phyla at their class and genus level. The most abundant genera in the seed sludge were *Rhogostoma* (18.9%), followed by *Telotrochidium* (12.4%), *Opisthonecta* (4.8%) and *Epistylis* (2.6%), which belongs to the Oligohymenophorea class. Other peritrich ciliates genus such as *Pseudovorticella, Vorticella* and *Vorticellides* were in abundance between 0.1 and 0.7%. Most of the target genera were reduced over time in the laboratory-scale samples, i.e., *Telotrochidium* to 2.7% and *Epistylis* to 0.2 % in the sample taken on day 104 - which was during the likely copper contamination in the AGS_*E. coli* reactor. Notably, on day 160, when the AGS_*E. coli* system was again stable, *Telotrochidium* highly recovered to 23.2% and *Epistylis* to 1.8%. Regarding the genus *Rhogostoma,* irrespective of the circumstances, the genus was prevalent and highly abundant in the AGS_*E. coli* reactor. It appeared to be slightly affected on day 104 (46.2 %) for the undesired copper addition (Madoni et al., 1992), but fully recovered on day 160 (56.5%). Regarding the AGS_MS2 reactor sample on day 90, apart from the genus *Rhogostoma* (with a relative abundance of 29%), *Telotrochidium* (1.7%), and *Epistylis* to 0.2% were as conventional as the AGS_ *E. coli* on day 104. For the rest of the community, their relative abundance was between 0.01 and 0.25%, but more diverse than the AGS_ *E. coli* reactor.

3.4. Predation recorded using fluorescent staining

Figure 8 shows pictures obtained when recording the ingestion of the *E. coli* by attached ciliated protozoa using dsGreen labelled *E. coli* bacteria. The pictures confirmed *E. coli* predation by stalked ciliates. The stained bacteria were visible inside the vacuoles of the organisms, and the *E. coli* bacteria were also visible embedded in the granular sludge matrix. Upon ingestion, the bacteria were concentrated, but the technique was not suitable for quantification of the partitioning of *E. coli* bacteria between supernatant fraction, attached to the granular surface or inside of the granular biomass.

3.5. Attachment kinetics

Attachment tests were carried out with the surrogates (*E. coli* bacteria and MS2 bacteriophages). The attachment kinetics are represented in Figure 9. Two concentrations were tested versus the exposure time. Regardless of the concentration and the target organism, a speedy attachment occurred onto the granules in the first 15 min (Figure 9a and Figure 9b). Moreover, as can be observed in Figure 9c and Figure 9d, sharper curves can be observed

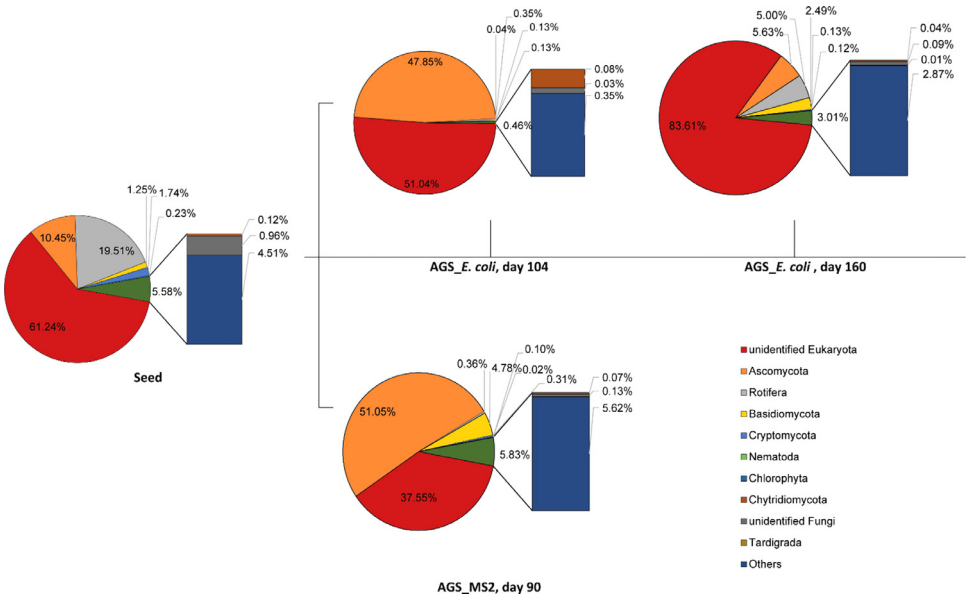

Figure 6. The relative abundance of the ten more frequent genome sequences at the phylum-level (a) and genus-level (a) taxonomy for the seed sludge, AGS_*E. coli* and AGS_MS2 reactor samples.

for the lower concentrations of both organisms, meaning that a relative faster attachment occurred when compared with the more concentrated samples.

3.6. Contribution of settling in the AGS reactors to the removal of the faecal surrogates

Additional tests were carried out to better understand the surrogates' removal in the liquid phase shown in the curve of Figure 3b, specifically from the point measured in the supernatant at the end of the aeration to the mixed effluent. For *E. coli* bacteria, it seems settling responsible for a 0.5 Log_{10} removal; while for the MS2 bacteriophage, an increase of 0.1 Log_{10} is measured. Results from the settling test (Figure 10) shows that regardless of the initial concentrations (2.0×10^5 and 5.3×10^7 CFU/100 mL for *E. coli*, and 2.4×10^3 and 4.7×10^9 PFU/100 mL for MS2), no differences were measured in the liquid bulk right after settling, nor in the liquid fraction of the treated effluent. Therefore, settling forces were discarded as factors to explain the variations in the long term reactors.

4. Discussion

4.1. Reactor performance

The AGS reactors were both under operational conditions that triggered quick granular formation and an efficient reactor performance (De Kreuk and Van Loosdrecht, 2004; Adav et al., 2008). Overall, both studied systems showed comparable performances to previous reports using similar substrates (Winkler et al., 2011). The addition to the *E. coli* and MS2 after day 47 for the AGS_*E. coli* reactor and day 69 for the AGS_MS2 reactor did not affect the general reactor performance. The measured water quality parameters shown in Figure 1 confirmed healthy systems with low effluent COD values, and high P-release after the anaerobic feeding of the AGS_*E. coli* and AGS_MS2 reactor, respectively. Except for the low NH_4-N removal in the AGS_*E. coli* reactor (from day 95 to 120), good conversion of NH_4-N to NO_2-N and NO_3-N during aeration was observed in both systems. The low NH_4-N removal in the AGS_*E. coli* reactor was most likely caused by the unexpected presence of copper in the system. Copper can be toxic for ammonia-oxidising bacteria (Sato et al., 1988) and other organisms such protozoa, which can also interfere in the nitrification process (Lee and Welander, 1994). After copper was excluded from the influent, the system recovered and showed a good performance until the end of the study.

4.2. Fate of the target surrogates during the operational conditions of the long-term reactors

The overall *E. coli* removal efficiency (2.2 Log_{10}) calculated for the AGS_*E. coli* reactor was within the range previously reported for *E. coli* in full-scale AGS systems. However, the MS2 bacteriophage removal (0.7 Log_{10}) was lower than reported (Barrios-Hernández et al., 2020b). It is worth mentioning that full-scale AGS systems contain both a large granular sludge fraction and a smaller flocculent sludge fraction (Pronk et al., 2015b). Ali et al. (2019) have shown variances in the bacterial assembly depending on the different size of the aggregates, including flocs, large and small granular fractions. Reactors here studied contained only granular sludge, potentially impacting the removal of the faecal surrogates. Therefore, the fate of the two different faecal surrogates (*E. coli* and MS2 bacteriophages) focused on evaluating only the contribution of the granular fraction during different operational stages of two AGS systems to better understand their influence on the pathogen surrogates removals.

4.2.1. Faecal surrogates removals during the anaerobic plug flow feeding

In this study, an average reduction of 0.3 Log_{10} was observed in the liquid fraction for the faecal surrogates during the anaerobic stage in both AGS_*E. coli* and AGS_MS2 reactors. In the AGS systems, the influent is fed in a plug-flow mode, causing a concentration gradient from high to low in the water phase over the granular bed. A high concentration of the faecal surrogates present in the influent at the feeding point/bottom of the reactor was expected, which is diluted with the "clean" water at the top part of the reactor after feeding. Chong et al. (2012) and Vymazal (2005) have shown that coliforms survive longer in anaerobic environments; thus, due to the fully anaerobic conditions, and the short time the feeding takes, the faecal surrogates' decay was

Class	Genus	Seed	AGS_*E. coli* (day 104)	AGS_*E. coli* (day 160)	AGS_MS2 (day 90)
Mediophyceae	*Stephanodiscus*	0.00%	0.01%	0.01%	0.04%
Craspedida	*Monosiga*	0.02%	0.00%	0.00%	0.02%
	Salpingoeca	0.02%	0.00%	0.00%	0.01%
Hyphochytriomycetes	*Rhizidiomyces*	0.37%	0.01%	0.00%	0.04%
Ichthyophonae	*LKM51*	0.02%	0.01%	0.02%	0.06%
Labyrinthulomycetes	*Sorodiplophrys*	0.05%	0.02%	0.05%	0.56%
	unidentified_Labyrinthulomycetes	0.01%	0.00%	0.00%	0.05%
Litostomatea	*Entodinium*	0.00%	0.00%	0.27%	0.02%
	Acineria	0.00%	0.00%	0.00%	0.03%
Oligohymenophorea	*Telotrochidium*	12.39%	2.72%	23.24%	1.70%
	Epistylis	2.61%	0.17%	1.75%	0.20%
	Opisthonecta	4.80%	0.49%	0.09%	0.25%
	Pseudovorticella	0.68%	0.12%	0.10%	0.15%
	Vorticella	0.55%	0.04%	0.17%	0.07%
	Colpidium	0.00%	0.04%	0.06%	0.09%
	Paramecium	0.04%	0.02%	0.02%	0.18%
	unidentified_Conthreep	0.50%	0.01%	0.03%	0.02%
Peronosporomycetes	*unidentified_Peronosporomycetes*	1.20%	0.03%	0.12%	2.13%
	Pythium	0.05%	0.01%	0.06%	0.18%
Phyllopharyngea	*Tokophrya*	1.99%	0.02%	0.03%	0.08%
	unidentified_Conthreep	0.27%	0.02%	0.05%	0.02%
	Discophrya	0.01%	0.00%	0.00%	0.04%
	Acineta	0.00%	0.00%	0.01%	0.02%
	Trochilia	0.00%	0.01%	0.00%	0.01%
Prostomatea	*Prorodon*	0.23%	0.02%	0.01%	0.17%
Dinophyceae	*Scrippsiella*	0.00%	0.01%	0.00%	0.00%
Spirotrichea	*Aspidisca*	1.50%	0.09%	0.01%	0.12%
	Rimostrombidium	0.00%	0.000%	0.00%	0.00%
	Euplotes	0.00%	0.000%	0.00%	0.00%
Ichthyophonae	*Anurofeca*	0.01%	0.000%	0.01%	0.01%
unidentified_Eukaryota	*Rhogostoma*	18.87%	46.194%	56.50%	29.03%
	Blastocystis	0.01%	0.01%	0.01%	0.01%
Armophorea	*Metopus*	0.002%	0.00%	0.00%	0.00%
Plagiopylea	*Trimyema*	0.00%	0.000%	0.002%	0.002%

Figure 7. Heat map at the genus-level for AGS_*E. coli* and AGS_MS2. Genera comprised of the most abundant protozoa phyla.

negligible. Furthermore, the batch attachment tests showed that irrespective of the organisms, the *E. coli* bacteria and the MS2 bacteriophage quickly attach and saturate the granular surface when passing through the granular media (Figure 9). They kept an equilibrium concentration between the granules and the supernatant fraction, as can also be observed in Figure 4. In case sludge waste occurs at this stage, as is practice in full-scale AGS treatment plants, a high concentration of surrogates (approximately 10^5 CFU/ 100 mL and 10^7 PFU/ 100 mL) would leave the system via the mixed liquor, a combination of the sludge and supernatant fraction here studied (Corpuz et al., 2020; Martín-Díaz et al., 2020). Sludge treatment and dewatering will result in (additional) removal of pathogens (Zeng et al., 2019; Zhang et al., 2020). Therefore, based on previous findings, the minor reduction reported during the anaerobic period can be attained to a constant dilution effect of the influent with the remaining reactor media during steady-state conditions.

4.2.2. Faecal surrogate removal during the aeration phase

During the aeration phase, the self-immobilised granular bed is continuously mixed, and granules are exposed to all the components remaining in the liquid bulk. Besides providing the right oxygen concentration in the systems (Lochmatter et al., 2013), aeration provides high shear stress helping to form round-shaped granules (Van Loosdrecht et al., 1995). This aerated phase is meaningful for some organisms that are oxygen depended such as protozoa (Fenchel, 2014).

Protozoa play a major role in wastewater treatment technologies (McKinney and Gram, 1956; Madoni, 2011; Amaral et al., 2018); they are unicellular, heterotrophs and eukaryotic organisms fed either by the absorption of dissolved nutrients or the ingestion of particulate matter, including bacteria or organism present during the assimilation (Nisbet, 1984). Pauli, et al. (2001) stated that protozoa could bind to bacterial conglomerates, such as flocs. In our laboratory-scale AGS reactors, the sludge beds were almost completely formed of granules. Smaller granules were quickly dis-

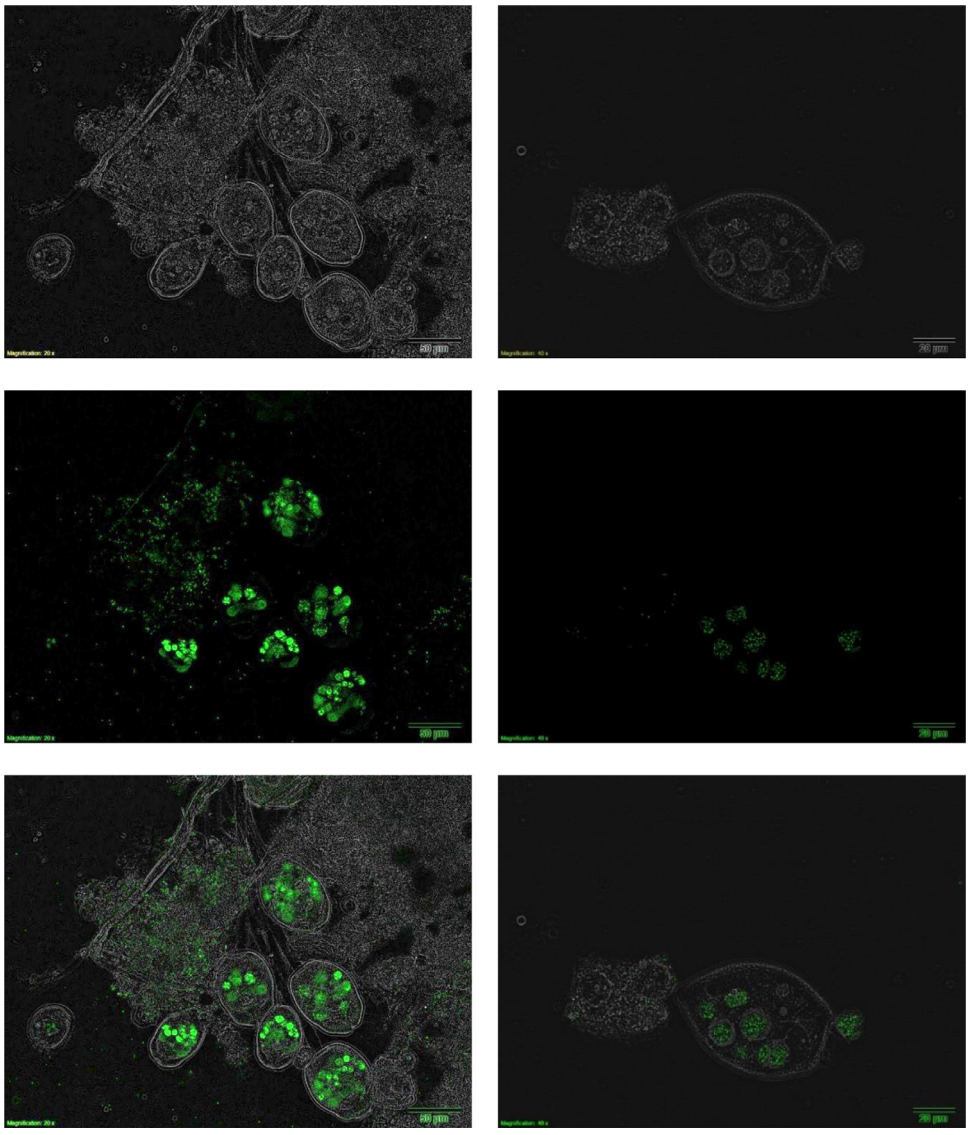

Figure 8. Phase-contrast picture (top), fluorescence microscopy (dsGreen) (middle) and their overlap (bottom), showing the ingestion of the bacteria by a colony of stalked ciliates (left side) and a single stalked ciliate (right side). The bar represents 50 and 20µm, respectively.

charged during the effluent withdrawal inducing free-living metazoans such as nematodes, tardigrades, and rotifers to wash out (Figure 6).

At the beginning of this study, a reduction of the protozoa was measured using a microscope; however, once *E. coli* was added to the AGS_*E. coli* system, a bloom of stalked ciliated protozoa attached to the granular surface occurred. This tendency has been previously reported in laboratory-scale systems fed on particulate material (de Kreuk et al., 2010; Barrios-Hernández et al., 2020a), and generally in full-scale wastewater treatment systems (Stevik et al., 2004). As can be seen in Figure 5a, a higher *E. coli* removal was detected when a higher abundance of attached ciliated protozoa was microscopically observed. Ciliates are a dominant class in wastewater treatment systems (Curds, 1973; Varma et al., 1975; Dubber and Gray, 2011); they move through cilia and are subdivided into three categories, free-swimming, crawling and attached organisms. Examples of this are the free-swimming genera *Telotrochidium* and *Opisthonecta*, and the attached ciliates *Epistylis, Pseudovorticella, Vorticella* and *Vorticellides*, which were part of the Ciliophora phylum-level found in this study (Gao et al., 2016; Leal et al., 2016; Adl et al., 2019). The free-living genus *Rhogostoma* from the Cercozoa phylum showed to be the most abundant in the AGS_*E. coli* reactor after both 104 (46%) and 160 (57%) operational days. It grows quickly under controlled (laboratory) conditions (Belar, 1921). Öztoprak et al. (2020) describe this genus as a bacteria predator with a high diversity of clades, able to colonise a variety of habitats, including wastewater matrices. Some species such as the *R. micra* are related to debris and bacteria (Howe et al., 2011), others (*R. epiphylla*) have been recognized as food selective with an affinity to predate yeast from the Ascomycota and Basidiomicota phyla (Dumack et al., 2017); organisms that were also present during steady conditions of the AGS_*E. coli* reactor (Figure 6).

Concerning the protozoa filter-feeding process called phagocytosis (Berman, 2012), the feeding starts by generating a water current, concentrating the particulate matter present in the liquid bulk while retaining the particles in size between 0.3 and 5 µm (Mallory et al., 1983; Lynn, 2008), it includes our *E. coli* (1-2 µm). The process continues with the intake of the retained particulate matter in vacuoles. This intake was recorded in the individual batch tests when using fluorescently labelled *E. coli* (Figure 8). Phagocytosis was anticipated to occur only during aeration. Bac-

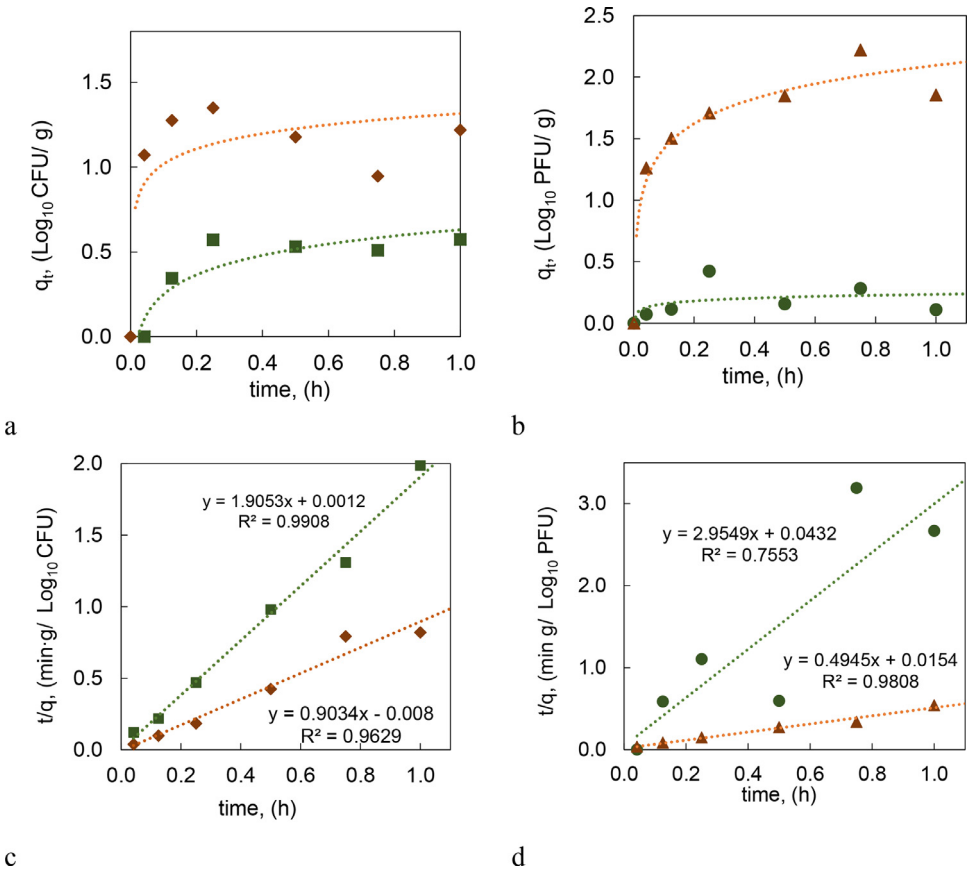

Figure 9. Attachment kinetics for: a) *E. coli* and b) MS2 bacteriophages. Linearized pseudo-second order kinetics for c) *E. coli* and d) MS2 bacteriophages. Fitted lines and equations are shown for each data set. Initial concentrations: *E. coli* 1.3 × 10^7 CFU/ 100 mL (■) and 2.5 × 10^9 CFU/ 100 mL (♦); MS2 bacteriophages 1.6 × 10^6 PFU/ 100 mL (●) and 5.5 × 10^9 PFU/ 100 mL (▲).

terivorous commonly found in anoxic environments such as *Metopus* and *Caenomorpha* from the Armophorea class were not present in the samples (Hirakata et al., 2016). Interestedly, the anaerobic ciliates *Trimyema* (Plagiopylea) occurred but in a very low taxonomic abundance (0.002%) when the AGS_*E. coli* system was stable at day 160 (Schulz et al., 1990). Fenchel (2014) stated that some other protozoa could also adapt and sustain their growth under oxygen limitations. It applies to *Euplotes* and *Rimostrombidium* from the Spirotrichea class. However, such genera were negligible in the studied systems. Matsunaga et al. (2014) reported a greater diversity of uncultured eukaryotes, including phylogenetic affiliations found in this study. Overall, our results were consistent with eukaryotic molecular diversity studies using 18S rRNA gene analysis in different sewage systems.

For the MS2 bacteriophages, no significant changes were recorded when the bacteriophage was added to the reactor. Bacteriophages, in general, are very selective on their host (Saha and Mukherjee, 2019). The host specificity of MS2 bacteriophage used in this study depended mostly on *E. coli* F-pili (Berzin et al., 1974; Salas and de Vega, 2008). Therefore, infections of bacteria forming the granules (AOB, NOB and PAOs) were not expected and not measured based on the physicochemical reactor performance (Zhang et al., 2013). Therefore, due to lack of favourable conditions for reproducing the MS2 bacteriophage, including a low host range of bacteria (Marks and Sharp, 2000; Khan et al., 2002) and slow infection cycles (Hantula et al., 1991), cell lysis was assumed negligible.

Indeed, the MS2 bacteriophages addition did not induce the same stalked ciliated protozoa bloom rate as in the AGS_*E. coli* reactor. As a particle, MS2 bacteriophage (27 nm) is much smaller than the *E. coli* bacteria (1 by 2 μm) (Kuzmanovic et al., 2003). Besides the low rate bacteria erosion expected from the granules (De Kreuk and Van Loosdrecht, 2004), and the lack of particulate material or any other bacteria in the synthetic influent, resulted in the reduced protozoa growth; indirectly affecting the MS2 bacteriophages removal. Overall, bacterivorous genera such as the free-swimming *Telotrochidium* (1.7%) and stalked *Epistylis* (0.2%) occurred. However, their relative abundance was lower compared with the values found for the AGS_*E. coli* reactor, which were 23% and 2%, respectively. The genus *Rhogostoma* (29%), which was also the most abundant organism found in the AGS_MS2 reactor, potentially grew by predating such free bacteria and fungi derived from the sludge granules, little contributing to the viral surrogates' removal. Deng et al. (2014) reported that the free-living *Salpingoeca* (Craspedida) can use the phage as a potential carbon source by actively feeding on MS2. It coincides with the relative taxonomic occurrence of this flagellate in the AGS_MS2 reactor (0.01%) which was slightly lower than in the seed sludge (0.02%), but not abundant in the reactor fed only with *E. coli*. Hence, based on prey selection criteria, the protozoa feeding rate determined the grazing pressure on the added bacteriophages (Jürgens, 2007).

Regarding attachment, it was assumed that the granule surface reached an attachment equilibrium with the liquid bulk right after the anaerobic feeding. This assumption can be confirmed when looking at the long term experiments in Figure 4a and Figure 4b. The *E. coli* median concentrations of the granular fractions at the end of the aerobic phase kept the same order of magnitude than the previous phase, about 10^5 CFU/100 mL for *E. coli* and 10^7 PFU/ 100 mL for MS2. Therefore, recognising the role of the protozoa in our laboratory-scale systems, the main removal mechanism during

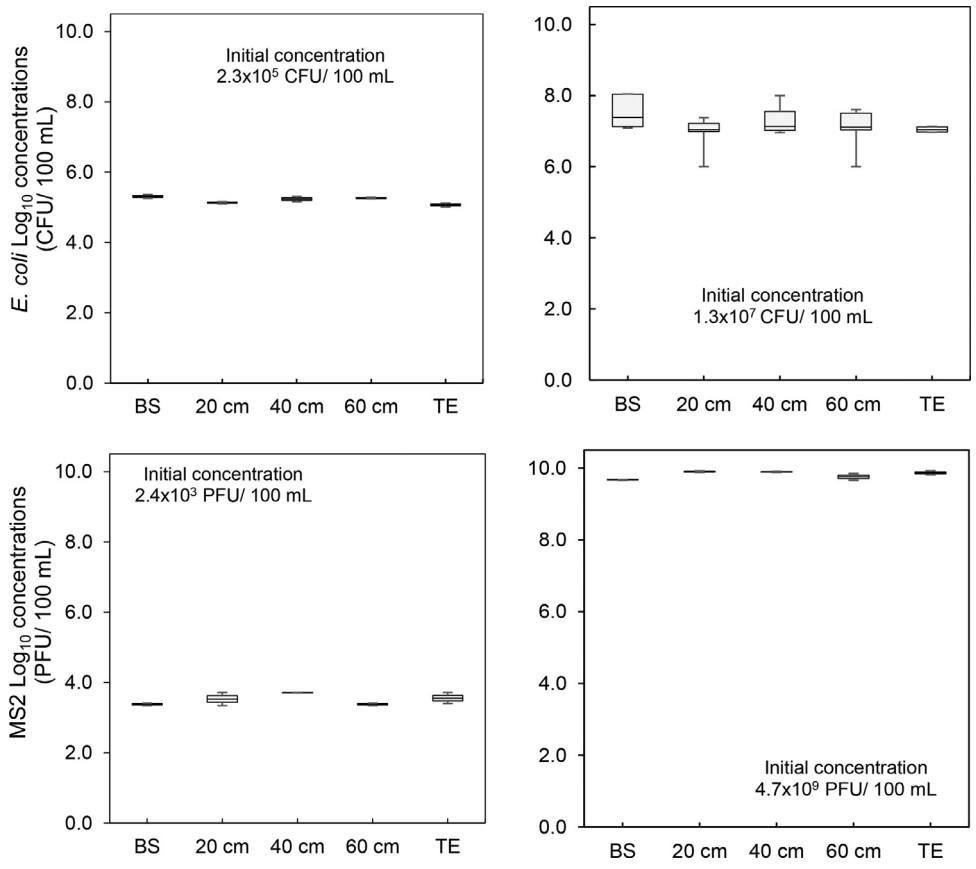

Figure 10. *E. coli* and MS2 counts obtained from the settling batch tests. Concentrations correspond to the medians of liquid bulk before settling occurred (BS) at 20, 40 and 60 cm height from the discharge point and treated effluent (TE).

aeration can be documented as a one-phase process. On average, 1 Log_{10} *E. coli* and 0.6 Log_{10} MS2 bacteriophages were measured during aeration by filter-feeding protozoa (van der Drift et al., 1977; Mallory et al., 1983) which for the system fed only with MS2 were less abundant than in the AGS_*E. coli* reactor.

4.3. Contribution of the settling in the removal of the faecal surrogates

The settling batch tests executed in the additional column reactor showed that settling forces do not contribute to removing any of the studied faecal surrogates in the liquid phase (Figure 10). In the laboratory-scale reactors, the treated effluent is rapidly separated from the biomass due to its high density (Beun et al., 2002). The surrogates initially attached to the granular surface settle along with the granules (Figure 4). Therefore, the effluent is a mixture of supernatant with high concentrations of suspended organisms and very small granules that did not settle during the short settling time (5 min). Such effluent composition explains the dynamic of the organism's depletion curve during settling time shown in Figure 3b; which for the *E. coli* seemed that settling is adding to the overall removal. But for MS2 bacteriophage, similar concentrations between effluent and the liquid fraction after aeration were observed. Therefore, the variations found in the organism's depletion curve were based on the composition of the effluent samples, but not caused by any selection pressure.

4.4. The relevance of the findings for future applications

In this study, in the laboratory granular sludge system fed only with synthetic wastewater, the removal of bacteria was higher than the removal of bacteriophages. The granules were saturated with high amounts of the surrogates (*E. coli* and MS2), achieving a saturation point during steady-state. As previously mentioned in Section 4.2.1, such surrogates and actual pathogenic organisms will potentially abandon the system via waste sludge (Guzman et al., 2007; Goberna et al., 2018). In AGS full-scale systems, sludge waste normally occurs by a selection pressure that will discharge a high amount of flocculent sludge not commonly found in laboratory-scale reactors (van Dijk et al., 2020). Some physical properties such as cell mobility (Pratt and Kolter, 1998), opposite charge attraction (Tay et al., 2000), hydrophobicity (Tay et al., 2001; Liu et al., 2003), and type of substrate added (Pronk et al., 2015a) might influence their attachment. *E. coli* and MS2 bacteriophages are considered good indicators of actual bacterial and viral pathogens in wastewater (Dias et al., 2018). Their removal in full-scale AGS systems has been reported and compared with parallel CAS systems (Barrios-Hernández et al., 2020b), along with the dynamics of antibiotic resistance genes (Pallares-Vega et al., 2020). So far, further research of the behaviour of actual bacterial, viral and eukaryotic pathogenic organism in the AGS system is missing. Full-scale AGS systems are fed with complex substrates, they develop a significant flocculent sludge fraction derived from the influent suspended solids. According to Ali et al. (2019), bacteria entering the system via the influent may end up in that flocculent fraction of the AGS systems. Therefore, the influence of the flocculent fraction on the bacterial and viral pathogen removal dynamics can be further considered. Influent raw wastewater can also have an impact on the diversity of the eukaryotic structures (Hirakata et al., 2019). In our study, protozoa predation was the dominant mechanism to the actual removal of the surrogates. As protozoa are ideal grazers, their abundance and diversity could help to achieve pathogens

and antibiotic-resistant bacteria removal. A characterization of the protozoa community in a full-scale AGS system may help to better understand the pathogen removal dynamics of the AGS systems.

5. Conclusions

This study investigated the effects of physical and biological mechanisms on removing two important water quality surrogates, *E. coli* bacteria and MS2 bacteriophages, in AGS systems. Regardless of the organism, the bacterial and viral surrogates quickly attached to the granular surface saturating the granules during steady-state conditions. Therefore, physical removal plays a role when sludge waste occurs. During aeration, the *E. coli* bacteria and MS2 bacteriophages were reduced to approximately 1 and 0.6 Log_{10}, respectively. Protozoa predation was the main contributor to the removal of *E. coli* during aeration. The 18S rRNA sequence analysis confirmed the occurrence of the genera *Pseudovorticella, Vorticella* and *Vorticellides*, which are attached ciliates from the phylum ciliophoran. A higher abundance of free-living genus *Rhogostoma* and the free-swimming ciliates *Telotrochidium* were also found. In the system fed with MS2 bacteriophages, a similar eukaryotic community was observed, although at much lower amounts. Bacteriophages removal was low in the system spiked only with MS2. In full-scale AGS systems protozoa growth on the granular sludge fraction can significantly contribute to the removal of bacteria from the influent. The flocculent sludge fraction is responsible for further reducing bacterial numbers and bacteriophages and needs more attention in future research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.116992.

References

Adav, S.S., Lee, D.J., Show, K.Y., Tay, J.H., 2008. Aerobic granular sludge: recent advances. Biotechnol Adv 26 (5), 411–423.
Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., 2019. Revisions to the classification, nomenclature, and diversity of eukaryotes. Journal of Eukaryotic Microbiology 66 (1), 4–119.
Ali, M., Wang, Z., Salam, K.W., Hari, A.R., Pronk, M., Van Loosdrecht, M.C., Saikaly, P.E., 2019. Importance of species sorting and immigration on the bacterial assembly of different-sized aggregates in a full-scale aerobic granular sludge plant. Environmental science & technology 53 (14), 8291–8301.
Amaral, A.L., Leal, C.S., Vaz, A.I., Vieira, J.C., Quinteiro, A.C., Costa, M.L., Castro, L.M., 2018. Use of chemometric analyses to assess biological wastewater treatment plants by protozoa and metazoa monitoring. Environmental Monitoring and Assessment 190 (9), 497.
Amarasiri, M., Kitajima, M., Nguyen, T.H., Okabe, S., Sano, D., 2017. Bacteriophage removal efficiency as a validation and operational monitoring tool for virus reduction in wastewater reclamation: review. Water Research 121, 258–269.
Anon, 2000a. ISO 9308-1:2000. International Organization for Standarisation, Geneva, Switzerland.
Anon, 2000b. ISO 10705-1. International Organization for Standarisation, Geneva, Switzerland.
APHA, 2012. Standard Methods for the Examination of Water and Wastewater. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.
Bales, R.C., Li, S., Maguire, K.M., Yahya, M.T. and Gerba, C.P.J.W.R.R. 1993. MS-2 and poliovirus transport in porous media: Hydrophobic effects and chemical perturbations. 29(4), 957-963.
Barrios-Hernández, M.L., Buenaño-Vargas, C., García, H., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2020a. Effect of the co-treatment of synthetic faecal sludge and wastewater in an aerobic granular sludge system. Science of The Total Environment, 140480.
Barrios-Hernández, M.L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2020b. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. Water Research X 6, 100040.
Belar, K., 1921. Untersuchungen über Thecamöben der Chlamydophrys-Gruppe. Arch. Protistenkd 43, 287–354.
Bengtsson, S., de Blois, M., Wilén, B.-M., Gustavsson, D., 2018. Treatment of municipal wastewater with aerobic granular sludge. Critical Reviews in Environmental Science and Technology 48 (2), 119–166.
Berman, J.J., 2012. Chapter 20 - Ciliophora (Ciliates). In: Berman, J.J. (Ed.), Taxonomic Guide to Infectious Diseases. Academic Press, Boston, pp. 111–112.
Berzin, V., Rosenthal, G., Gren, E.J., 1974. Cellular macromolecule synthesis in Escherichia coli infected with bacteriophage MS2. Eur J Biochem 45 (1), 233–242.
Beun, J., Van Loosdrecht, M., Heijnen, J., 2002. Aerobic granulation in a sequencing batch airlift reactor. Water Research 36 (3), 702–712.
Chao, A., 1984. Nonparametric estimation of the number of classes in a population. Scandinavian Journal of statistics 265–270.
Chong, S., Sen, T.K., Kayaalp, A., Ang, H.M., 2012. The performance enhancements of upflow anaerobic sludge blanket (UASB) reactors for domestic sludge treatment – A State-of-the-art review. Water Research 46 (11), 3434–3470.
Corpuz, M.V.A., Buonerba, A., Vigliotta, G., Zarra, T., Ballesteros, F., Campiglia, P., Belgiorno, V., Korshin, G., Naddeo, V., 2020. Viruses in wastewater: occurrence, abundance and detection methods. Science of The Total Environment 745, 140910.
Curds, C.R.J.A.R.i.M. 1982. The ecology and role of protozoa in aerobic sewage treatment processes. 36(1), 27-28.
Curds, C.R.J.A.Z. 1973. The role of protozoa in the activated-sludge process. 13(1), 161-169.
De Kreuk, M., Van Loosdrecht, M., 2004. Selection of slow growing organisms as a means for improving aerobic granular sludge stability. Water Science & Technology 49 (11), 9–17.
de Kreuk, M., Kishida, N., Tsuneda, S., Van Loosdrecht, M., 2010. Behavior of polymeric substrates in an aerobic granular sludge system. Water Research 44 (20), 5929–5938.
de Kreuk, M.K., Kishida, N., van Loosdrecht, M.C.M., 2007. Aerobic granular sludge – state of the art. Water Science and Technology 55 (8-9), 75.
de Sousa Rollemberg, S.L., Mendes Barros, A.R., Milen Firmino, P.I., Bezerra dos Santos, A., 2018. Aerobic granular sludge: Cultivation parameters and removal mechanisms. Bioresource Technology 270, 678–688.
Deng, L., Krauss, S., Feichtmayer, J., Hofmann, R., Arndt, H., Griebler, C., 2014. Grazing of heterotrophic flagellates on viruses is driven by feeding behaviour. Environmental Microbiology Reports 6 (4), 325–330.
Dias, D., Passos, R., Von Sperling, M., 2017. A review of bacterial indicator disinfection mechanisms in waste stabilisation ponds. Bio/Technology 16 (3), 517–539.
Dias, E., Ebdon, J., Taylor, H., 2018. The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems. Water Research 129, 172–179.
Dubber, D. and Gray, N.J.W.r. 2011. The effect of anoxia and anaerobia on ciliate community in biological nutrient removal systems using laboratory-scale sequencing batch reactors (SBRs). 45(6), 2213-2226.
Dumack, K., Flues, S., Hermanns, K., Bonkowski, M., 2017. Rhogostomidae (Cercozoa) from soils, roots and plant leaves (Arabidopsis thaliana): Description of Rhogostoma epiphylla sp. nov. and R. cylindrica sp. nov. European Journal of Protistology 60, 76–86.
Fenchel, T., 2014. Protozoa and Oxygen. Acta protozoologica 53, 3–12.
Gao, F., Warren, A., Zhang, Q., Gong, J., Miao, M., Sun, P., Xu, D., Huang, J., Yi, Z., Song, W., 2016. The all-data-based evolutionary hypothesis of ciliated protists with a revised classification of the phylum Ciliophora (Eukaryota, Alveolata). Scientific Reports 6, 24874.
Goberna, M., Simón, P., Hernández, M.T., García, C., 2018. Prokaryotic communities and potential pathogens in sewage sludge: Response to wastewaster origin, loading rate and treatment technology. Science of The Total Environment 615, 360–368.
González, J.M., Suttle, C.A., 1993. Grazing by marine nanoflagellates on viruses and virus-sized particles: ingestion and digestion. Marine Ecology Progress Series 1–10.
Guzman, C., Jofre, J., Montemayor, M., Lucena, F., 2007. Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. Journal of Applied Microbiology 103 (6), 2420–2429.
Hantula, J., Kurki, A., Vuoriranta, P., Bamford, D.H., 1991. Ecology of bacteriophages infecting activated sludge bacteria. Appl. Environ. Microbiol. 57 (8), 2147–2151.
Hendricks, D.W., Post, F.J., Khairnar, D.R., 1979. Adsorption of bacteria on soils: ex-

periments, thermodynamic rationale, and application. Water, Air, and Soil Pollution 12 (2), 219–232.
Hirakata, Y., Oshiki, M., Kuroda, K., Hatamoto, M., Kubota, K., Yamaguchi, T., Harada, H., Araki, N., 2016. Effects of predation by protists on prokaryotic community function, structure, and diversity in anaerobic granular sludge. Microbes and environments ME16067.
Hirakata, Y., Hatamoto, M., Oshiki, M., Watari, T., Kuroda, K., Araki, N., Yamaguchi, T., 2019. Temporal variation of eukaryotic community structures in UASB reactor treating domestic sewage as revealed by 18S rRNA gene sequencing. Scientific Reports 9 (1), 12783.
Howe, A.T., Bass, D., Scoble, J.M., Lewis, R., Vickerman, K., Arndt, H., Cavalier–Smith, T., 2011. Novel Cultured Protists Identify Deep-branching Environmental DNA Clades of Cercozoa: New Genera Tremula, Micrometopion, Minimassisteria, Nudifila, Peregrinia. Protist 162 (2), 332–372.
Jürgens, K., 2007. Predation on Bacteria and Bacterial Resistance Mechanisms: Comparative AspectsAmong Different Predator Groups in Aquatic Systems. In: Jurkevitch, E. (Ed.), Predatory Prokaryotes: Biology, Ecology and Evolution. Springer, Berlin Heidelberg; Berlin, Heidelberg, pp. 57–92.
Khan, M.A., Satoh, H., Katayama, H., Kurisu, F., Mino, T., 2002. Bacteriophages isolated from activated sludge processes and their polyvalency. Water research 36 (13), 3364–3370.
Kim, B.-R., Shin, J., Guevarra, R., Lee, J.H., Kim, D.W., Seol, K.-H., Lee, J.-H., Kim, H.B., Isaacson, R.E., 2017. Deciphering diversity indices for a better understanding of microbial communities. J Microbiol Biotechnol 27 (12), 2089–2093.
Kuzmanovic, D.A., Elashvili, I., Wick, C., O'Connell, C., Krueger, S., 2003. Bacteriophage MS2: Molecular Weight and Spatial Distribution of the Protein and RNA Components by Small-Angle Neutron Scattering and Virus Counting. Structure 11 (11), 1339–1348.
Leal, C., Amaral, A.L., de Lourdes Costa, M., 2016. Microbial-based evaluation of foaming events in full-scale wastewater treatment plants by microscopy survey and quantitative image analysis. Environmental Science and Pollution Research 23 (15), 15638–15650.
Lee, N.M., Welander, T., 1994. Influence of Predators on Nitrification in Aerobic Biofilm Processes. Water Science and Technology 29 (7), 355–363.
Lin, Y., de Kreuk, M., Van Loosdrecht, M., Adin, A., 2010. Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot–plant. Water research 44 (11), 3355–3364.
Liu, Y., Tay, J.-H., 2002. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. Water Research 36 (7), 1653–1665.
Liu, Y., Yang, S.-F., Liu, Q.-S., Tay, J.-H., 2003. The role of cell hydrophobicity in the formation of aerobic granules 46 (4), 0270–0274.
Lochmatter, S., Gonzalez-Gil, G., Holliger, C., 2013. Optimized aeration strategies for nitrogen and phosphorus removal with aerobic granular sludge. Water Research 47 (16), 6187–6197.
Lucena, F., Duran, A., Moron, A., Calderon, E., Campos, C., Gantzer, C., Skraber, S., Jofre, J., 2004. Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. Journal of Applied Microbiology 97 (5), 1069–1076.
Lynn, D., 2008. The ciliated protozoa: characterization, classification, and guide to the literature. Springer Science & Business Media.
Madoni, P., Esteban, G., Gorbi, G., 1992. Acute toxicity of cadmium, copper, mercury, and zinc to ciliates from activated sludge plants. Bulletin of environmental contamination and toxicology 49 (6), 900–905.
Madoni, P., 1994. Estimates of ciliated protozoa biomass in activated sludge and biofilm. Bioresource Technology 48 (3), 245–249.
Madoni, P., 2011. Protozoa in wastewater treatment processes: a minireview. Italian Journal of Zoology 78 (1), 3–11.
Mallory, L., Yuk, C., Liang, L., Alexander, M., 1983. Alternative prey: a mechanism for elimination of bacterial species by protozoa. Applied and Environmental Microbiology 46 (5), 1073–1079.
Marks, T., Sharp, R., 2000. Bacteriophages and biotechnology: a review. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology 75 (1), 6–17.
Martín-Díaz, J., Lucena, F., Blanch, A.R., Jofre, J., 2020. Review: Indicator bacteriophages in sludge, biosolids, sediments and soils. Environmental Research 182, 109133.
Matsunaga, K., Kubota, K., Harada, H., 2014. Molecular diversity of eukaryotes in municipal wastewater treatment processes as revealed by 18S rRNA gene analysis. Microbes and environments 29 (4), 401–407.
McKinney, R.E., Gram, A., 1956. Protozoa and activated sludge. Sewage and Industrial Wastes 28 (10), 1219–1231.
Nancharaiah, Y.V., Kiran Kumar Reddy, G., 2018. Aerobic granular sludge technology: Mechanisms of granulation and biotechnological applications. Bioresource Technology 247, 1128–1143.
Nisbet, B., 1984. Nutrition and feeding strategies in protozoa. Croom Helm, London.
Öztoprak, H., Walden, S., Heger, T., Bonkowski, M., Dumack, K., 2020. What Drives the Diversity of the Most Abundant Terrestrial Cercozoan Family (Rhogostomidae, Cercozoa, Rhizaria)? Microorganisms 8 (8), 1123.
Pallares-Vega, R., Leal, L.H., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C., Weissbrodt, D.G., Schmitt, H., 2020. Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. Water Research, 116752.
Pratt, L.A. and Kolter, R.J.M.m. 1998. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. 30(2), 285-293.
Pronk, M., Abbas, B., Al-zuhairy, S.H.K., Kraan, R., Kleerebezem, R., van Loosdrecht, M.C.M, 2015a. Effect and behaviour of different substrates in relation to the formation of aerobic granular sludge. Applied Microbiology and Biotechnology 99 (12), 5257–5268.
Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M., 2015b. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Research 84, 207–217.
Pronk, M., Giesen, A., Thompson, A., Robertson, S., van Loosdrecht, M.C.M., 2017. Aerobic granular biomass technology: advancements in design, applications and further developments. Water Practice and Technology 12 (4), 987–996.
Saha, D., Mukherjee, R., 2019. Ameliorating the antimicrobial resistance crisis: phage therapy. IUBMB Life 71 (7), 781–790.
Salas, M., de Vega, M., 2008. Replication of Bacterial Viruses. In: Mahy, B.W.J., Van Regenmortel, M.H.V. (Eds.), Encyclopedia of Virology. Academic Press, Oxford, pp. 399–406.
Sato, C., Leung, S.W., Schnoor, J.L., 1988. Toxic response of Nitrosomonas europaea to copper in inorganic medium and wastewater. Water Research 22 (9), 1117–1127.
Schulz, S., Wagener, S., Pfennig, N., 1990. Utilization of various chemotrophic and phototrophic bacteria as food by the anaerobic ciliate Trimyema compressum. European Journal of Protistology 26 (2), 122–131.
Schwarzenbeck, N., Erley, R., Wilderer, P.J.W.S., 2004. Technology. Aerobic granular sludge in an SBR-system treating wastewater rich in particulate matter 49 (11-12), 41–46.
Scoullos, I.M., Adhikari, S., Lopez Vazquez, C.M., van de Vossenberg, J., Brdjanovic, D., 2019. Inactivation of indicator organisms on different surfaces after urban floods. Science of The Total Environment, 135456.
Shi, Y., Liu, Y., 2021. Evolution of extracellular polymeric substances (EPS) in aerobic sludge granulation: Composition, adherence and viscoelastic properties. Chemosphere 262, 128033.
Simonin, J.-P.J.C.E.J. 2016. On the comparison of pseudo-first order and pseudo-second order rate laws in the modeling of adsorption kinetics. 300, 254-263.
Stevik, T.K., Aa, K., Ausland, G., Hanssen, J.F., 2004. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: a review. Water Research 38 (6), 1355–1367.
Szabó, E., Liébana, R., Hermansson, M., Modin, O., Persson, F., Wilén, B.-M., 2017. Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates. Frontiers in microbiology 8, 770.
Tay, J.-H., Xu, H.-L., Teo, K.-C., 2000. Molecular mechanism of granulation. I: H+ translocation-dehydration theory 126 (5), 403–410.
Tay, J.H., Liu, Q.S. and Liu, Y.J.J.o.A.M. 2001. Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. 91(1), 168-175.
Thwaites, B.J., Short, M.D., Stuetz, R.M., Reeve, P.J., Alvarez Gaitan, J.-P., Dinesh, N., van den Akker, B., 2018. Comparing the performance of aerobic granular sludge versus conventional activated sludge for microbial log removal and effluent quality: implications for water reuse. Water Research 145, 442–452.
van der Drift, C., van Seggelen, E., Stumm, C., Hol, W., Tuinte, J., 1977. Removal of *Escherichia coli* in wastewater by activated sludge. Applied and environmental microbiology 34 (3), 315–319.
van Dijk, E.J.H., Pronk, M., van Loosdrecht, M.C.M., 2020. A settling model for full-scale aerobic granular sludge. Water Research 186, 116135.
Van Loosdrecht, M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L., Heijnen, J., 1995. Biofilm structures. Water Science and Technology 32 (8), 35.
Varma, M.M., Finley, H.E. and Bennett, G.H.J.J. 1975. Population dynamics of protozoa in wastewater. 85-92.
Vishniac, W., Santer, M., 1957. The thiobacilli. Bacteriological reviews 21 (3), 195.
Vymazal, J., 2005. Removal of enteric bacteria in constructed treatment wetlands with emergent macrophytes: a review. Journal of Environmental Science and Health 40 (6-7), 1355–1367.
Weber, S., Ludwig, W., Schleifer, K.-H., Fried, J., 2007. Microbial composition and structure of aerobic granular sewage biofilms. Applied and Environmental Microbiology 73 (19), 6233–6240.
Winkler, M.-K., Bassin, J., Kleerebezem, R., De Bruin, L., Van den Brand, T., Van Loosdrecht, M., 2011. Selective sludge removal in a segregated aerobic granular biomass system as a strategy to control PAO–GAO competition at high temperatures. Water research 45 (11), 3291–3299.
Winkler, M.-K.H., Kleerebezem, R., de Bruin, L.M.M., Verheijen, P.J.T., Abbas, B., Habermacher, J., van Loosdrecht, M.C.M., 2013. Microbial diversity differences within aerobic granular sludge and activated sludge flocs. Applied Microbiology and Biotechnology 97 (16), 7447–7458.
Zeng, Q., Zan, F., Hao, T., Biswal, B.K., Lin, S., van Loosdrecht, M.C.M., Chen, G., 2019. Electrochemical pretreatment for stabilization of waste activated sludge: Simultaneously enhancing dewaterability, inactivating pathogens and mitigating hydrogen sulfide. Water Research 166, 115035.
Zhang, L., Chen, Y., Ma, C., Liu, L., Pan, J., Li, B., Wu, X., Wang, Q., 2020. Improving heavy metals removal, dewaterability and pathogen removal of waste activated sludge using enhanced chemical leaching. Journal of Cleaner Production 271, 122512.
Zhang, Y., Hunt, H.K., Hu, Z., 2013. Application of bacteriophages to selectively remove Pseudomonas aeruginosa in water and wastewater filtration systems. Water Research 47 (13), 4507–4518.